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The genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase are expressed differentially in petunia leaves

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ABSTRACT

We have isolated five members of the multigene family encoding the small subunit (rbcS) of ribulose-1,5-bisphosphate carboxylase in petunia and examined their expression in petunia leaves. Of the five rbcS genes, two (ssu11A and ssu8) are expressed at high levels in petunia leaves. Northern analysis using gene specific oligonucleotide probes revealed that ssu11A accounts for 40% of the total rbcS transcripts in petunia leaves while ssu8 accounts for 4 to 5% of the total rbcS transcripts. Structural comparisons of ssu8 and ssu11A revealed that the coding sequence of ssu8 is interrupted by three introns, while the coding sequence of ssu11A is interrupted by two introns. The positions of the first two introns are identical, the third intron in ssu8 is located in a highly conserved region of the protein. The 5' and 3' flanking sequences of ssu11A are highly homologous to the 5' and 3' flanking sequences of ssu8. S1 nuclease mapping was used to locate the start of transcription of ssu8 and ssu11A and showed that ssu8 mRNA leader differs in sequence from the ssu11A mRNA leader.

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase-oxygenase catalyzes the carboxylation of ribulose-1,5-bisphosphate which represents the first reaction in photosynthetic carbon dioxide fixation. The enzyme is present in the chloroplasts of higher plants and is composed of eight large subunits (rbcL) of Mr~55,000 and eight small subunits (rbcS) of Mr ~ 14,000 (1). The rbcL is chloroplast encoded and is synthesized within the organelle (2,3). The rbcS is nuclear encoded, it is synthesized on cytoplasmic ribosomes and then transported into chloroplasts where it is processed into the mature form by cleavage of the transit peptide (4,5,6). The rbcS is encoded by a multigene family in higher plants (7,8). Several members of this gene family are expressed (9) and their expression is regulated by light (10,11) and is mediated by the light receptor phytochrome (12).

There is evidence that one of the pea rbcS genes is expressed in an organ-specific manner (13). This gene is expressed predominantly in leaf

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cells and is not expressed or expressed at lower levels in other organs of the plant (13).

In petunia, a multigene family which consists of at least eight different members, encodes the rbcS (14). Dunsmuir *et al.* (15) have isolated three different rbcS cDNA clones indicating that at least three different genes are transcribed in petunia leaves. They have classified the rbcS genes into three different families based on their homology to the cDNA clones. Two of these gene families contain single genes and one contains six genes (14).

In this report, we describe the cloning and expression of five different rbcS genes from petunia. The steady state levels of RNA were examined for two of these genes expressed at high levels in petunia leaves and were shown to differ 10-fold. We present the nucleotide sequence of both of these genes and attempt to analyze the differences in their expression by comparing their structure and nucleotide sequences.

MATERIALS AND METHODS

Plant Material

The Petunia (Mitchell) strain is a doubled haploid produced from a hybrid between Petunia hybrida and Petunia axillaris. VR is an F1 hybrid Petunia hybrida (violet 23 x red 51) produced in the genetics department at the University of Amsterdam. The plants were grown under greenhouse conditions.

Construction of Petunia Genomic Library

High molecular weight DNA was isolated from a cell suspension culture of petunia Mitchell diploid cell line (MP4) as described by Shure *et al.*(16). A recombinant phage library was prepared from a partial MboI digest of this DNA following the procedures of Maniatis *et al.* (17) using λMG14 as a cloning vector. λMG14 contains the left arm of λ1059, the right arm of charon30 and a 13.0 kb stuffer fragment of yeast DNA containing multiple EcoRI sites (G. Graham and M. Olson, personal communication). A cDNA clone, pSS15, which encodes the entire 123 amino acids of the mature pea rbcS polypeptide (18) was used as a hybridization probe to screen the petunia library. One recombinant was isolated from this library and shown to contain two rbcS genes, ssu11A and ssu11B. The 1.2 kb and 2.3 kb EcoRI fragments spanning the ssu11A gene were purified by preparative polyacrylamide gel electrophoresis and used to screen a library of petunia genomic DNA constructed as follows. High molecular weight DNA was isolated from a cell suspension culture of petunia Mitchell diploid cell line (MP4-G), digested to completion with BamHI and ligated to the arms of λMG14 using the procedures of Maniatis et al (17). Phage DNA was isolated using glycerol gradients (17).

Nucleotide Sequence Analysis

A 1.8 kb EcoRI fragment and a 1.8 kb HindIII fragment of the ssu8 clone (see Figure 1) were subcloned in pUC8 and designated pMON9558 and pMON9559, respectively. Likewise, 1.2 kb and 2.3 kb EcoRI fragments of ssu11A (see Figure 1) were subcloned into pUC9 and designated pMON9502 and pMON9503, respectively. DNA sequencing was carried out by the method of Maxam and Gilbert (19).

RNA Isolation

Leaves were harvested from mature petunia plants and frozen in liquid nitrogen. Total RNA from petunia leaves was prepared as described by Goldberg *et al.* (20). Total RNA was then dissolved in water and precipitated twice with 2M LiCl. Poly A^{+} RNA was selected by oligo-dT cellulose chromatography (21).

S1 Nuclease Mapping

A 1.6 kb EcoRI-HinfI fragment from the 5' end of ssu8 was labelled at its 5' ends, digested with DdeI and the 430 bp DdeI-HinfI fragment was hybridized with 3 μg leaf poly A^{+} RNA. Likewise, a 800 bp EcoRI-PvuII fragment from ssu11A was labelled at its 5' ends, digested with HindIII and Hybridized with 3 μg leaf poly A^{+} RNA. The hybridization and S1 nuclease digestion conditions were exactly as described by Tumer *et al.* (22).

Figure 1. Comparison of the restriction maps of ssu11A and ssu8 clones. The 1.2kb (pMON9502) and the 2.3kb (pMON9503) EcoRI fragments of ssu11A were subcloned in pUC9 and the 1.8kb EcoRI (pMON9558) and the 1.8kb HindIII (pMON9559) fragments of ssu8 were subcloned in pUC8. A restriction map of the lambda clone λ11 is shown. The position of the two rbcS genes within the petunia DNA is indicated. The direction of transcription of each gene is shown by the arrows.

```
   1  ATGTTATGGACAATATAAGGAAGCAAACAGTCTAGCTATCACTAGTTGGAACTTCTAAATCATAAATACT
  71  TGGGAAAACAAAAAAAGAAAAATTATATACATAAAAATGGTTTGCAAAAGGACCTAAAAAGAATTAAGTT
 141  ACCTCGATCACACATTCATATCCACTTCCTACTCCATATCGGATGAGATAAGATTACTAAGTGCTTCCAC
 211  GTGGCACCTCCTTTGTGGTGACATAATGAAGAGGGTCTTAGCTCCAAAAATACATTTCCAACCTTTCATG
 281  TGTGGATATTAAATTTGTAATATCAAGAACCACATAATCCAATGGTTAGCTTTACTCCAAGATGAGGTTA
 351  GTTGATTTTTGTCCGTTAGATATGTGAAATATGTAAAAACCTTATCATTATATAAAGGGTGTGGTGGGCA
 421  ATACAAAAGTCAGTGTGAAGTGTTTAAAGGAAAAAGCTTTGGAAGAAGCAAAAATCTTCTAACTATGGCT
                                                                      M  A
 491  TCCTCTGTGATTTCCTCTGCAGCTGTTGCTACTCGCACTAATGTGGCTCAAGCTAGCATGGTTGCACCTT
      S  S  V  I  S  S  A  A  V  A  T  R  T  N  V  A  Q  A  S  M  V  A  P  F
 561  TTAATGGTCTTAAGTCTGCTGTCTCCTTCCCAGTTTCAAGCAAGCAAAACCTTGACATCACTTCCATTGC
        N  G  L  K  S  A  V  S  F  P  V  S  S  K  Q  N  L  D  I  T  S  I  A
 631  TAGCAATGGTGGAAGAGTCCAATGCATGCAGGTACTTACTTTACATTATCACTACAGTTTAGCTTGTGAT
       S  N  G  G  R  V  Q  C  M  Q
 701  AGTAAGTGAATTATCAGTATTACTAGGTTAGTATTTACTAGCAATACTTATCATAAAAATATATTATGTA
 771  ATTACTCGGAACTTGAAACTCGAACAATCGAATTATAGCATTAATATATAGTAATAATCATGATGGAAAT
 841  GACAAATATTGTGCACCTTATATATCGTCCCAAAAGTTTAGAACTAGAACTGAATTCAAGATTTACGGTT
 911  AGTGAGTGCATAATAGGACCTACTTAGTGTTATCATGTATTTTGAACTTTTCTTTGTGATGTGTTCAATT
 981  GAAGTCACAGATAAAGCCAAGTACTCTAGATCTGATTATGCTAATTAAAACATGTTAGTGTCTAGTCAGA
1051  TCGAAAATTTAAACTACTAGTAGTTTGGAATCATTAATGTCATTTTGTACGAATTTGATTTTTTTTCCTC
1121  ACACTTCACAATCTTCAATACTAATTAAGTTCAGAGAGGAGATAGTAGGTATGAATTCATCAGTTGCTGC
1191  CTTACATAGGTCTTGCATATTAGCTAGTACTAATTGTATCACTTAAAATTCTCTGTTGAGTATAGGTGTG
                                                                     V  W
1261  GCCCCCATATGGCAAGAAGAAGTACGAGACTCTCTCATACCTTCCTGATTTAACCGACGAGCAATTGCTC
       P  P  Y  G  K  K  K  Y  E  T  L  S  Y  L  P  D  L  T  D  E  Q  L  L
1331  AAGGAGATTGAGTACCTTTTGAACAAGGGATGGGTTCCTTGCTTGGAATTTGAGACTGAGGTCAAGATCT
      K  E  I  E  Y  L  L  N  K  G  W  V  P  C  L  E  F  E  T  E
1401  TTCTTTTTTCTTTTAAACATTCGCTACTATAAACACGTTTAACATGATTATCAAAGTTATATTTTCGACA
1471  TCACGAGGAAGTATCTTAATGTCTTGTTTTATATGTGCAGCACGGATTTGTCTACCGTGAATACCACGCC
                                               H  G  F  V  Y  R  E  Y  H  A
1541  TCACCTAGATACTATGATGGAAGGTTAGTAATGGATGTACACAATTTTTATTTTATTTATTGTACCAGCA
      S  P  R  Y  Y  D  G  R
1611  GCTGTGTGAAAATACTGAAAATTAATACAAATACTATTGAGCTCATACTTTCAAAGAACTTAGAACATTG
1681  AACACGTCATAATTAAATTTTGAATATGCTCTGTGATAGGTACTGGACCATGTGGAAGTTGCCCATGTTT
                                               Y  W  T  M  W  K  L  P  M  F
1751  GGGTGCACTGATGCAACTCAGGTGTTGGGTGAGCTCCAAGAGGCCAAGAAGGCTTACCCTAATGCATGGA
      G  C  T  D  A  T  Q  V  L  G  E  L  Q  E  A  K  K  A  Y  P  N  A  W  I
1821  TCAGAATCATCGGATTCGACAACGTCCGTCAAGTGCAATGCATCAGTTTCATTGCCTACAAGCCACCAGG
        R  I  I  G  F  D  N  V  R  Q  V  Q  C  I  S  F  I  A  Y  K  P  P  G
1891  CTTCTAAGTTATATTAGGACAGCTTACCCTATGTATTTAGGGGCAGTTTGTTTTAATTGTACTTAGGTTG
       F  *
1961  TTTACTTGTTTTTTATTGAAAACTGTTTCATTTCCTTATTTAATTTCTATTCGGTGTATGTTTTTGGATT
2031  CCAACCAAGTTATGAGAACTAATAATGATAATTTGGTGCTTTGTTTGTACATTTGTTGGTTGAGAGTCTT
2101  GTGGCCTATATAAGTTTTATCTTGATAATATCTCCTATCCTAAATGCAAATGGTCCACACTCCACATTGC
2171  ACCAGTAACATCCCCCTCCCCCCGGACTATCCTTTCATATTATGAAAGGCATTCAATCATTTCTTGAATT
2241  TC
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Northern analysis

Poly A^+ RNA from petunia leaves was fractionated on a 1.3% formaldehyde-agarose gel (17) transferred to nitrocellulose and hybridized with the oligonucleotide probes as follows.

Oligonucleotide Hybridization

Oligonucleotides were synthesized by an automated DNA synthesizer from Applied Biosystems, Inc. The oligonucleotides were labelled with ^{32}P at their 5' ends using T4 polynucleotide kinase (23). The dissociation temperature (Td) of each probe was approximated by the formula 2°C (A+T) + 4°C (G+C) (23). Hybridizations were performed at 10°C below the Td of each probe in 6 x SSC (1 x SSC is 0.15M NaCl, 0.015M Na-citrate, pH 7.0), 10 x Denhardt's (1 x Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrollidone, 0.02% BSA) and 200 µg/ml *E. coli* tRNA. After hybridization, the filters were washed for 15 minutes at room temperature in 6 x SSC, and then in 6 x SSC for 5 minutes at 4°C below the Td of each probe. Filters were then dried at room temperature and autoradiographed.

RESULTS

Isolation of Recombinant Phage Containing Petunia rbcS Genes

Approximately 1.5 x 10^5 recombinant phage of the petunia genomic library were hybridized under reduced stringency to a pea rbcS cDNA probe. Phage DNA was extracted from 15 independent recombinants. These DNA's were tested for cross-homology to cloned soybean (30) and wheat (31) rbcS sequences by Southern blot analysis. One recombinant, λ11, hybridized under reduced stringency to both the soybean and wheat rbcS probes (data not shown). Restriction map analysis showed that λ11 encompasses approximately 20 kb of petunia genomic DNA. It contains two rbcS genes, 11A and 11B, which are closely linked and reside within an interval of approximately 2 kb (Figure 1). The complete coding sequence of 11A was mapped to two EcoRI fragments of 1.2 kb and 2.3 kb containing, respectively, the 5' and 3' ends of the gene. The 1.2 kb EcoRI fragment specific for the 5' end and the 2.3 kb EcoRI fragment specific for the 3' end of ssu11A were used as probes to screen a genomic bank

Figure 2. Nucleotide sequence of the petunia genomic subclone ssu8, encoding rbcS. The deduced amino acid sequence is shown below the DNA sequence. The nucleotide indicated by the arrow is the putative transcription initiation site of ssu8. The sequences which resemble signals for transcriptional regulation of other eukaryotic genes are underlined. The sequences in paranthesis in the 5' and 3' regions refer to the sequences used in synthesizing gene specific oligonucleotide probes for hybridization analysis.

```
   1  GAAGTAAACTTAATGAGCTTCCGCCACGTGGCACTATTTTTATGACTAATATCTTTTTCC

  61  TAACCTTCATGTGGCCATTAATTTAGTAATGTCAAGAACACATAATCCAATGGTTACAAT

 121  TCATCCAAGATGAGGCTTGTTTACTTGTATCCGTTAGATGTAGAGGTTATGTGAAAGCTT

 181  AACATTATATATAGAAGGGGGCACTATACATCAATAACCCTCTTGAAGCAAAGGTGGGAA

 241  AGGGAAA[CACAAAAATATAAGCTAACGAT]TCTTTAGCAATGGCTTCCTCAGTGATGTCCT
                                               M  A  S  S  V  M  S  S

 301  CAGCTGCAGTTGCCACAAGCACCAATGCTGCTCAAGCCAGCATGGTTGCACCCTTCACTG
        A  A  V  A  T  S  T  N  A  A  Q  A  S  M  V  A  P  F  T  G

 361  GCCTCAAGTCTGCAGCCTCCTTCCCTGTTTCCAGGAAACAGAACCTTGACATTACTTCCA
        L  K  S  A  A  S  F  P  V  S  R  K  Q  N  L  D  I  T  S  I

 421  TTGCTAGCAATGGTGGAAGAGTTCAATGCATGCAGGTACGTAACATATTAACTTATATGC
        A  S  N  G  G  R  V  Q  C  M  Q

 481  ATTATCAGTGTGGAGAATTTTTACACCAACCATTTGTAGCAAGTAACTTGCATTATTTTT

 541  TAGGTTGCACATTTTATGCGTGTAATAATAATGTAACGTATTAATAGTGACGTAACATTG

 601  TAAATATTGTAAATACCGTTTGCCTTTTACTTTAGTAAAAGTTAGTTCAATATTTTGTTT

 661  TGTGGGATGTAGGTGTGGCCACCATACGGCAAGAAGAAGTACGAAACTCTCTCATACCTT
                   V  W  P  P  Y  G  K  K  K  Y  E  T  L  S  Y  L

 721  CCTGATTTGACTGACGAGCAGCTCCTCAAGGAAATTGAGTACCTTTTGAACAAGGGATGG
      P  D  L  T  D  E  Q  L  L  K  E  I  E  Y  L  L  N  K  G  W

 781  GTTCCTTGCTTGGAATTCGAGACTGAGGTTAAGATCTATTTTTTATTGACTTTGCTACTA
      V  P  C  L  E  F  E  T  E

 841  TTAAGCAGAACTAATATGAGTTTAAATTATACATAGTCAATCCCTAACTAATTTGGGGAC

 901  GTAATTGATTTATTGAAAGAAGCTATCTTATGTAAATGTTTGGTTTTATATGTGTAGCAC
                                                                H

 961  GGATTCGTCTACCGTGAGTACCATGCATCTCCAAGGTACTATGATGGCAGGTACTGGACC
      G  F  V  Y  R  E  Y  H  A  S  P  R  Y  Y  D  G  R  Y  W  T

1021  ATGTGGAAGCTGCCCATGTTCGGGTGCACCGATGCCACCCAAGTCTTGGGTGAGCTCCAA
      M  W  K  L  P  M  F  G  C  T  D  A  T  Q  V  L  G  E  L  Q

1081  GAGGCCAAGAAGGCTTACCCCAATGCCTGGATCAGAATCATTGGATTCGACAACGTGCGT
      E  A  K  K  A  Y  P  N  A  W  I  R  I  I  G  F  D  N  V  R

1141  CAAGTGCAATGCATCAGTTTCATTGCCTACAAGCCCCCAGGCTACTAGATTT[CATTTTAA
      Q  V  Q  C  I  S  F  I  A  Y  K  P  P  G  Y  *

1201  AACAACCTATCCT]CTGTTTTTAGGGGCAATTTGCTGAATTATCCTTAGGTTTTTCCCTTA

1261  AAAAACTGCTTTTTTTCCTTTAGTTCTTTCTATTCCGTGTATGTTTTTGGATCC
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Figure 3. The nucleotide sequence and the deduced amino acid sequences for the petunia genomic subclone ssu11A. Upstream sequences corresponding to the 'CAT' and 'TATA' box regions are underlined. The putative transcription initiation site is overlined. The sequences in brackets in the 5' and 3' flanking regions refer to the sequences used in synthesizing gene specific oligonucleotide probes for hybridization analysis.

of BamHI digested petunia DNA. Approximately 1 x 10^5 recombinant phage were screened and fifteen positively hybridizing phage were isolated. The Southern analysis of the DNAs from these phage showed that three different members of the petunia rbcS gene family are represented by these clones. These clones designated λ2, λ5 and λ8 contain 17 kb, 6.6 kb and 15 kb BamHI fragments of petunia genomic DNA, respectively. λ11A which contains the previously

isolated 11A gene on a 17 kb BamHI fragment was also isolated from this library. Using the 5' and 3' specific fragments of the ssu11A gene, we determined the orientation of rbcS genes in these clones and showed that each clone contains an intact rbcS gene.

Structural Analysis of ssu8 and ssu11A Genes

In order to identify the highly expressed members of the isolated genes, poly A^+ RNA from petunia leaves was labelled with ^{32}P-ATP and hybridized to a Southern blot containing restriction enzyme digests of DNA from each of the four clones. Under high stringency hybridization (50% formamide, 48°C) and washing (70°C in 0.1 x SSC) conditions, the poly A^+ RNA probe hybridized strongly to the restriction fragments containing ssu8 and ssu11A genes, whereas ssu2 and ssu5 genes showed very low levels of hybridization (data not shown). The highly expressed ssu8 and ssu11A were therefore chosen for further analysis and their structures were compared at the nucleotide sequence level.

In order to delineate the detailed exon-intron structure and to unravel the complete coding information of ssu11A and ssu8 genes, we have determined their nucleotide sequence including portions of their 5' and 3' flanking sequences. Restriction maps of these two genes along with the restriction map of the lambda clone 11 are shown in Figure 1. Restriction map of the lambda clone 8 is identical to the previously published map of the 15 kb BamHI fragment of Φ30 (14). The complete nucleotide sequence of the ssu8 gene is shown in Figure 2. The nucleotide sequence of the rbcS coding sequence as well as 484 nucleotides of the 5' flanking and 344 nucleotides of the 3' flanking sequences are presented. The complete nucleotide sequence of the ssu11A is shown in Figure 3. The sequence of the rbcS coding region as well as 279 nucleotides of 5' flanking and 126 nucleotides of 3' flanking sequences are presented. The structure of the ssu8 gene deduced from the nucleotide sequence is compared with the structure of ssu11A gene in Figure 1. The most striking difference between the two genes is that ssu8 gene has three introns while ssu11A gene has two introns. The positions of the two introns in the ssu11A gene are conserved in the ssu8 gene. The third intron in ssu8 splits the arg codon at position 65 and is located in a highly conserved region of the protein. A rbcS gene containing three introns has been recently isolated from tobacco (25). The positions of introns in this gene are identical to those of the ssu8 gene. The nucleotide sequences of the ssu8 and ssu11A coding sequences are 89% identical. However, the conservation in the predicted amino acid sequences is much greater. They differ by five amino

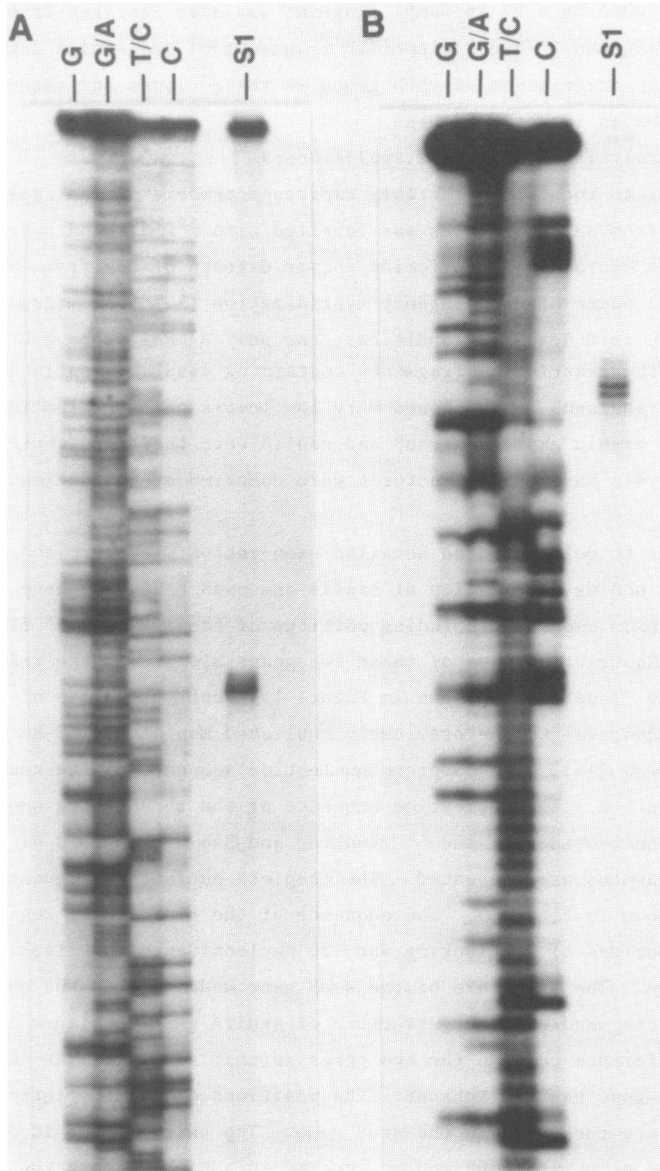

Figure 4. Identification of the start point of transcription of the ssu8 (A) and ssu11A (B) genes. The ssu8 probe was a 430 bp DdeI-HinfI fragment. The ssu11A probe was a 126 bp HindIII-PvuII fragment. Both probes were labelled at the 5' end with kinase, hybridized to petunia leaf poly A^{+} RNA and treated with S1 nuclease as described in the Materials and Methods. Sequencing reaction products of the DNA probes used are also shown.

acids in the transit peptide and by a single amino acid in the mature polypeptide sequence. The homology between the ssu8 gene and the structurally related tobacco rbcS gene is 86% at the nucleotide sequence level and 87% at the amino acid sequence level.

The 5' end of the ssu8 rbcS transcript was mapped by S1 nuclease mapping as described in Materials and Methods. The results shown in Figure 4 reveal that the transcription of the ssu8 gene begins at the A residue 53 nucleotides upstream of the start of translation. A sequence resembling the TATA box is located thirty-two nucleotides upstream of the transcription initiation site. The CAAT box is located 112 nucleotides upstream of the transcription initiation site. These sequences resemble the regulatory sequences found upstream of other eukaryotic genes (26).

The 5' end of the ssu11A mRNA was also determined by S1 nuclease analysis. (Figure 4) Based on the size of the S1 protected fragment, the 5' end of the transcript occurs 65-66 nucleotides upstream of the initiation codon, ATG. Twenty-five nucleotides 5' to the start of transcript is a "TATA" box-like sequence 'TATATATA'. At 110 nucleotides upstream of the 5' end of the rbcS transcript is the sequence 'CCAAT' which resembles the 'CAAT' box sequence located in the -70 to -80 region of other eukaryotic genes (Figure 3).

The comparison of the 5' flanking sequences of ssu8 and ssu11A genes shows a high degree of homology. The sequences around the TATA and the CAAT boxes, as well as sequences upstream of the CAAT box are 80% conserved. No conservation is evident, however, between the 5' leader sequences of the two transcripts. There is also striking homology in the 5' flanking region of the ssu8 and the tobacco rbcS genes. The conserved region extends further into the 5' flanking sequences and the overall homology between the ssu8 and the tobacco genes is 80% in this region. Another interesting feature of the rbcS genes is that they contain in their 5' flanking regions, sequences similar to the consensus sequences of viral and mammalian enhancer sequences (13, 25). Petunia ssu8 gene has four blocks of sequences in the 5' flanking region which exhibit homology to the sequence of the "core" enhancer element, $GTGG^{AAA}_{TTT}G$ (27). The enhancer like sequence GTGTGGATA (underlined in Figure 2) is also present in the tobacco and pea rbcS genes at approximately the same position (13, 25). This sequence is not present in the 5' flanking region of the ssu11A gene; however, a nine base pair sequence which differs by a single nucleotide is present within the first intron of ssu11A gene. The

Table I. A Comparison of the nucleotide sequence of the 5' flanking regions of petunia rbcS genes

Gene	Sequence
ssu11A	A C A C A A A A A T A T A A G C T A A C G A T T C T T T A G C A A T G
ssu8 (ssu301)	A A A G C T T T G G A A G A A G C A A A A A T C T T C T A A C T A T G
ssu611	A G G A G T C A T A G T G C A A T G A C C A T C A T A A A G C A A T G
ssu491	A G C T C A A G G G A A C C A A G G A T T T A T T T T C A G A A A T G
ssu112	A C C G C A A T A A C T T T T C T A A G G A T A T T T C A G C A A T G
ssu911	G T C A A G G G A A G C A A T A G C A A T T A T A T T T A G C A A T G

We have aligned the 5' flanking sequences of ssu11A and ssu8 with the 5' flanking sequences of ssu611, ssu491, ssu112 and ssu911 (28). Translation initiation codon of each rbcS gene and the sequences corresponding to the oligonucleotide probes are underlined.

significance of these sequences in the trancription of rbcS genes remains to be investigated.

We also find a high degree of homology between the 3' flanking sequences of the ssu8, ssu11A and tobacco rbcS genes. The region of homology starts immediately following the translation termination codon and extends into the 3' flanking sequences. The conservation between ssu8 and ssu11A genes in this region is 75% and between ssu8 and tobacco gene is 80%.

Differential Expression of rbcS Genes in Petunia Leaves

Our initial results indicated that there are differences in the relative expression of the individual rbcS genes in petunia leaves. We have examined the steady state levels of RNA for two highly expressed genes, ssu8 and ssu11A, using gene specific oligonucleotide probes.

Examination of the 5' and 3' flanking sequences of petunia rbcS genes showed that the leader sequences of the mRNAs were most divergent. We therefore selected our 5' probes from these regions. We have also synthesized probes complementary to the 3' flanking sequences of the rbcS mRNAs and used these along with the 5' probes to examine the expression of ssu8 and ssu11A genes.

In order to examine the expression of ssu8, a 22-mer oligonucleotide complementary to the 5' leader region of the mRNA was synthesized. Similarly, a 22-mer oligonucleotide complementary to the 5' leader of the ssu11A mRNA was synthesized. The sequences of ssu11A and ssu8 corresponding to these oligonucleotide probes are underlined in Table I and compared with the

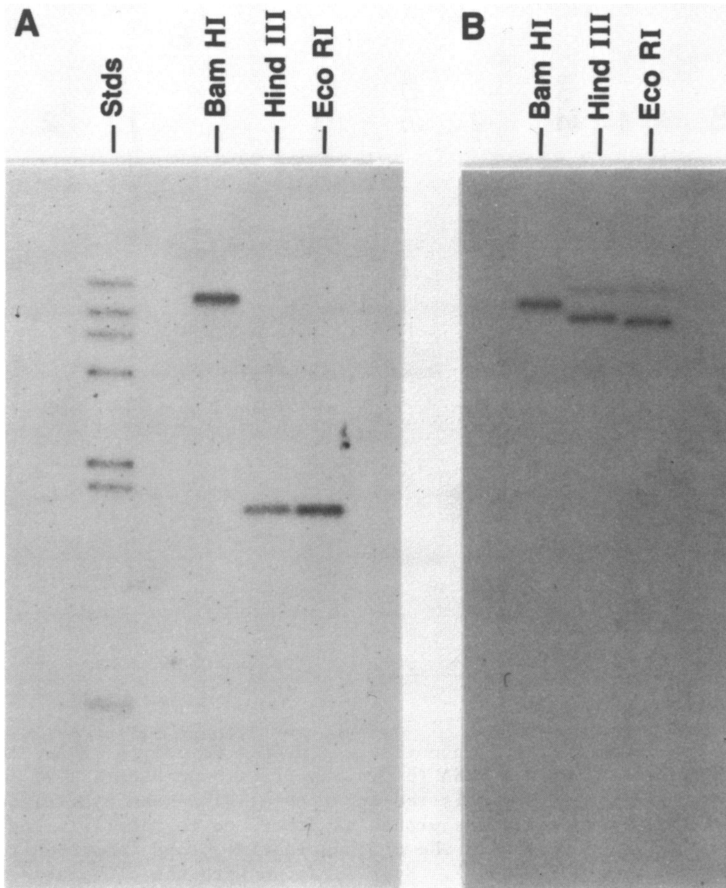

Figure 5. Hybridization of the oligonucleotide probes for ssu8 and ssu11A to the corresponding genomic clones. A. Hybrization of the oligonucleotide probe complementary to the 5' flanking sequences of ssu8 to BamHI, HindIII and EcoRI digests of λ8. B. Hybridization of the oligonucleotide probe complementary to the 3' flanking sequences of ssu11A to BamHI, HindIII and EcoRI digests of λ11A. ^{32}P-labelled HindIII fragments of λDNA were used as size markers.

sequences of ssu611, ssu491, ssu112 and ssu911 published by Dean et al. (28). The restriction map and the 5' and 3' flanking sequences of ssu8 are identical to the 5' and 3' flanking sequences of ssu301 (28). The sequences corresponding to the oligonucleotide probe for ssu8 are not homologous to the sequences of ssu11A or four other petunia rbcS genes shown in Table I. Likewise, the sequences corresponding to the oligonucleotide probe for ssu11A are not homologous to ssu8 (ssu301) or to the sequences of four other petunia rbcS genes (Table I). The sequences of the probes for ssu8 or ssu11A are also

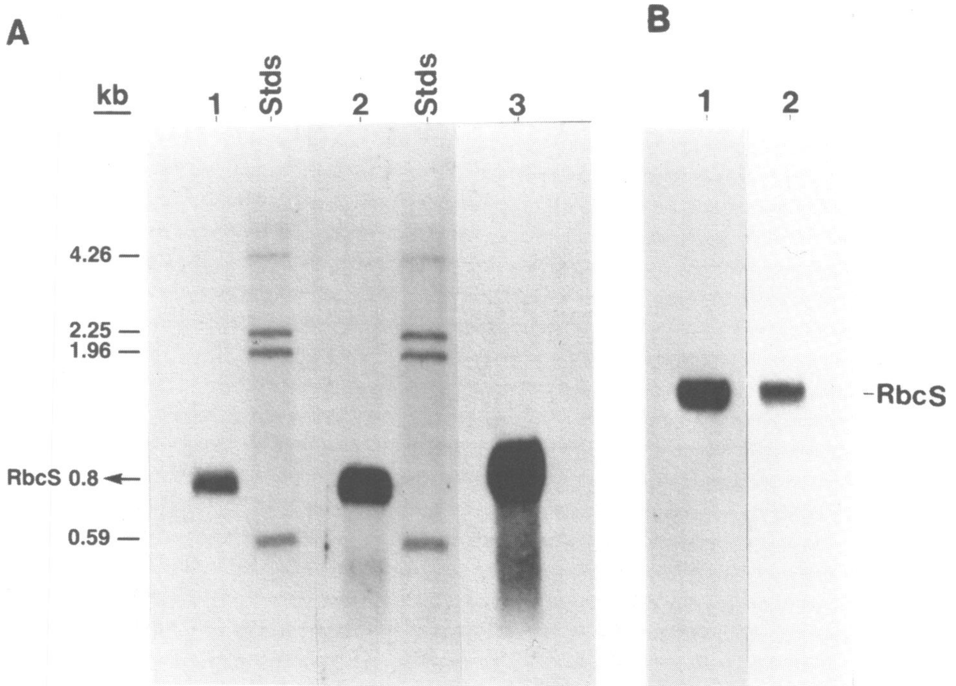

Figure 6. Hybridization of oligonucleotide probes to poly A^{+} RNA from petunia leaves. Petunia leaf poly A^{+} RNA (5 μg) was fractionated on a 1.3% formaldehyde-agarose gel, transferred to nitrocellulose and hybridized with the ^{32}P-labelled oligonucleotide probes as described in Materials and Methods. (A) Lane 1. Hybridization with the oligonucleotide probe complementary to 5' flanking region of ssu8. Lane 2. Hybridization with the oligonucleotide probe complementary to 5' flanking region of ssu11A. Lane 3. Hybridization with the oligonucleotide probe complementary to 3' coding sequences of petunia rbcS genes. (B) Lane 1. Hybridization with the oligonucleotide probe complementary to 3' flanking region of ssu11A. Lane 2. Hybridization with the oligonucleotide probe complementary to 3' flanking region of ssu8.

not homologous to the sequence of ssu231 (C. Dean, personal communication). Furthermore, of the five rbcS genomic clones we have isolated, ssu8 probe hybridizes only to ssu8 and ssu11A probe hybridizes only to ssu11A. The ssu11A probe has a GC content of 27% and the ssu8 probe has a GC content of 32%. These probes were labelled with ^{32}P using T4 polynucleotide kinase. To ensure that the two probes were of equal specific activity, they were first analyzed on 20% acrylamide-urea gels and the fractions which had similar specific activities were separately hybridized to DNA blots containing restriction digests of genomic clones corresponding to ssu8 or ssu11A. We

Table II. DNA sequences of synthetic oligonucleotide probes for petunia rbcS genes.

Gene	Probe	DNA Sequence
ssu11A 5' probe	NTS5	5' A T C G T T A G C T T A T A T T T T T G T G 3'
ssu8 5' probe	NTS3	5' T T A G A A G A T T T T T G C T T C T T C C 3'
common probe	NTS4	5' G T A G G C A A T G A A A C T G A T G C A 3'
ssu11A 3' probe	NT11A	5' A G G A T A G G T T G T T T T A A A A T G 3'
ssu8 3' probe	NTS6	5' A A G C T G T C C T A A T A T A A C T T A 3'

have repeated this analysis for the probes complementary to the 3' flanking sequences of ssu8 and ssu11A and for the combinations of 5' specific probe for ssu8 and 3' specific probe for ssu11A and for the 3' specific probe for ssu8 and 5' specific probe for ssu11A to show that the probes used had equal specific activities and hybridization efficiencies.

Figure 5 shows a representative experiment in which the oligonucleotide probe complementary to 5' flanking sequences of ssu8 and the oligonucleotide probe complementary to 3' flanking sequences of ssu11A are hybridized to restriction digests of λ8 (A) and λ11A (B). As shown in Figure 5, both probes hybridized to their corresponding genomic clones with very similar efficiencies. The probes complementary to the 5' flanking sequences of ssu8 and 5' flanking sequences of ssu11A also hybridized to their corresponding genomic clones with very similar efficiencies.

To compare the levels of transcripts from ssu8 and ssu11A genes, oligonucleotide probes complementary to the 5' flanking sequences of ssu8 and ssu11A, prepared as described above, were hybridized to Northern blots of poly A^+ RNA isolated from the leaves of petunia plants. The results of this analysis, shown in Figure 6A, indicated that ssu11A probe hybridized more strongly to leaf poly A^+ RNA than the ssu8 probe. Quantitation of the hybridization signals by densitometric scanning indicated a 10-fold difference. When the oligonucleotide/RNA duplexes were washed at higher stringency, they still maintained a 10-fold difference in their intensities. We repeated this experiment using oligonucleotide probes complementary to the 3' flanking regions of the two genes. We used a 21-mer oligonucleotide

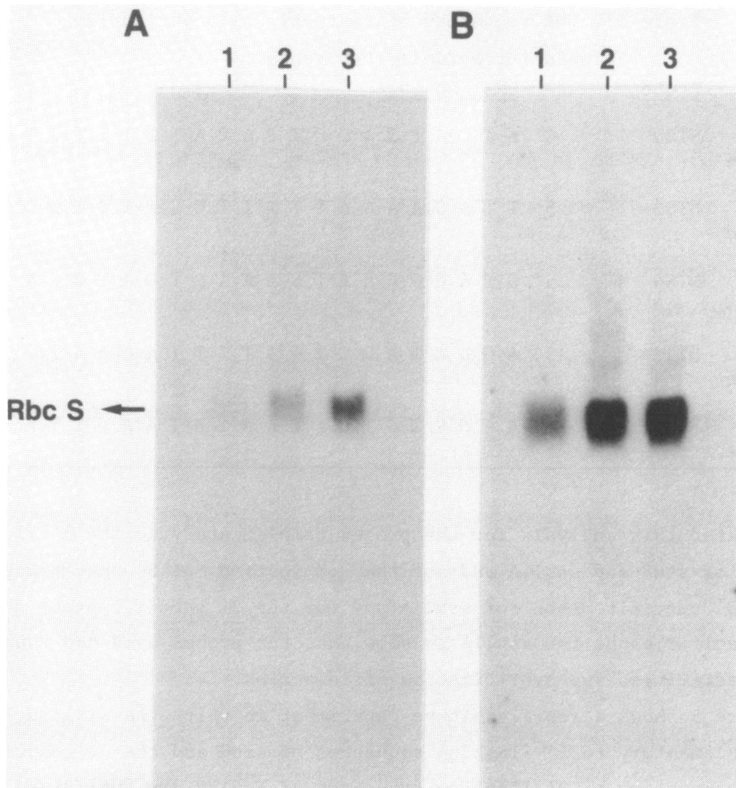

Figure 7. Hybridization of oligonucleotide probes to total RNA from petunia leaves. 50 μg of total RNA was fractionated on a 1.3% formaldehyde-agarose gel, transferred to nitrocellulose and hybridized with the oligonucleotide probes complementary to the 5' flanking sequences of ssu8 (A) and ssu11A (B), as described in the Materials and Methods. Lane 1. Poly A^+ RNA from petunia (Mitchell) leaves. Lane 2. Total RNA from petunia (Mitchell) leaves. Lane 3. Total RNA from petunia (VR) leaves.

complementary to the 3' flanking sequence of ssu8 and a 21-mer oligonucleotide complementary to the 3' flanking sequence of ssu11A (Table II see Figures 2 and 3 respectively, for exact location of these sequences). These probes differed in their GC content from the previous set of probes, thus their Td's and hybridization temperatures were different. Northern analysis using these probes confirmed our previous results and showed that petunia leaves contain higher levels of RNA for ssu11A than for ssu8 (Figure 6B). Upon quantitation of these signals by densitometric scanning, we again observed a 10-fold difference in the steady state levels of RNA specific for ssu8 and ssu11A.

In order to quantitate the levels of transcripts for these two genes

relative to the total rbcS transcripts, we used an oligonucleotide probe which is complementary to a coding sequence common to the three petunia rbcS cDNAs (15) and the two petunia rbcS genes (ssu8 and ssu11A) sequenced to date (NTS4, Table II). The results of Northern analysis, shown in Figure 6A, revealed that the transcription of ssu11A gene accounts for approximately 40% of the total rbcS transcripts while that of the ssu8 gene accounts for 4 to 5% of the total rbcS transcripts in petunia leaves.

We wanted to ensure that the differences in the hybridization intensities observed with ssu8 and ssu11A probes were not due to an artifact of our poly A^+ RNA preparations resulting from the differences in the lengths of polyA tails on rbcS mRNAs. We therefore repeated the Northern experiment using total RNA from petunia leaves. The blot shown in Figure 7 illustrates that the ssu11A transcripts are more abundant than the ssu8 transcripts. These results were obtained with Mitchell strain of petunia and were also found to be true for another petunia strain (VR) (Figure 7).

DISCUSSION

In higher plants, rbcS is encoded by several nuclear genes and at least some of these are expressed *in vivo* (9). To determine if individual members of this gene family are expressed differentially in petunia leaves, we isolated different members of this gene family and examined the expression of two rbcS genes which are expressed at high levels in petunia leaves.

We examined the expression of ssu8 and ssu11A genes, using oligonucleotide probes complementary to 5' or 3' flanking regions. Oligonucleotide probes were used since examination of the nucleotide sequences of the two genes revealed a high degree of homology in the coding (89%) as well as in the 5' and 3' flanking (80%) regions. The region which exhibited the least homology was the 5' leader of the two mRNAs. Hence, our 5' probes were selected from these regions and hybridizations were performed at temperatures such that the oligonucleotide/RNA hybrids would be stable only if base pairing were perfect (29).

The results of Northern analysis using gene-specific probes indicated that petunia leaves contained 10-fold higher levels of poly A^+ RNA specific for ssu11A than for ssu8. These results were confirmed using probes complementary to the 3' flanking regions of the two genes. By comparing the expression levels of these genes with the total expression level of petunia rbcS genes, we conclude that ssu11A accounts for 40% of the total rbcS gene expression while ssu8 accounts for 4% to 5% of the total rbcS gene expression

in petunia leaf tissue. We confirmed these results, using total RNA from petunia leaves and showed that ssu11A is expressed at higher levels in two different petunia strains examined (Mitchell and VR). The differences in the expression of these genes could be due to differences in the rates of transcription or alternatively, due to post-transcriptional modifications. Our experiments would not differentiate between these possibilities.

We have attempted to analyze the differences in the levels of expression of these genes by comparing their structure and nucleotide sequences. The structural organization of the two genes is different. The ssu8 gene has three introns while the ssu11A has two introns. This organization is different from pea or soybean where all the rbcS genes isolated contain two introns (13, 30), and from a wheat rbcS gene which has a single intron (31). The organization of ssu8 is similar to a tobacco rbcS gene which has three introns (25). The positions of the introns are identical between the two genes, however, the intron sizes and sequences vary. In addition to the similarity in organization, there is also a high degree of conservation in the coding as well as the 5' and 3' flanking regions between petunia ssu8 and the tobacco rbcS gene. This surprisingly high degree of homology suggests that these genes originated from the same ancestral gene and that this happened before the solanaceous plants diverged from one another. The level of expression of the tobacco rbcS gene has not been reported, thus we do not know if there is conservation at the level of gene expression between the structurally similar petunia and tobacco rbcS genes.

The sequences at the 3' flanking region of ssu8 and ssu11A are highly conserved. The extent of homology in this region is even greater between members of the same family. For example, ssu11A and another member of the same family, ssu231 differ by only two nucleotides in the 3' flanking region, although the 5' leader sequences of the two transcripts are quite different (C. Dean, personal communication). Thus, differences in the 3' flanking sequences of these genes might not be sufficient to account for the variability observed in the steady state levels of RNA.

In a recent report, Dean et al. have examined the levels of rbcS mRNA in petunia leaves and in other organs of the plant (28). They have concluded that ssu301 which is identical to ssu8 described in this report, accounts for 47% of the total rbcS gene expression in petunia leaves. The disagreement between their results and ours is partly due to the fact that they have estimated the relative expression levels by the relative abundance of rbcS cDNA clones they recovered. In addition, they have not recovered ssu11A from

their genomic library, thus they could not compare its expression level with that of ssu301.

The use of probes covering the 3' flanking sequences complicates their expression analysis because there is a high degree of homology in these regions between the rbcS genes. This homology is even greater between members of the same family such as ssu11A and ssu231 which are almost identical in the 3' flanking region (28). The use of oligonucleotide probes synthesized from regions which differ in sequence between these genes is therefore a more reliable method to estimate the expression of individual rbcS genes.

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